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(54) Title: ASSAY DEVICE AND METHOD OF DETECTING CHITIN

(57) Abstract

A method for detecting chitin-containing organisms such as fungi, yeast and insects in a sample attached to a solid phase, by detecting the presence of chitin by using enzymes which specifically bind chitin is disclosed. The method is preferably carried out by passing a sample which contains chitin through a membrane in order to capture and adhere the chitinous material on the membrane. A chitin-binding enzyme such as chitinase or lysozyme is added under conditions which allow it to bind to the chitin on the membrane. A signal generating compound is either directly conjugated to the chitinase (or lysozyme) or indirectly conjugated to either an anti-chitinase (or lysozyme) antibody or to an anti IgG antibody (which binds to the anti-chitinase antibody) to detect the chitin-chitinase complex. Reagents for the signal generating compound then are added to produce a detection signal which indicates the presence of the chitin. Kits which contain the necessary materials for carrying out the methods of the invention are also disclosed as are novel conjugates.

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ASSAY DEVICE AND METHOD OF DETECTING CHITIN

Field of the Invention

This invention relates to the field of assays to detect target substances, and particularly to assays to determine the presence of chitin and thereby determine the presence of organisms which contain chitin such as fungi, yeast, and insects in a sample.

Background

Infections caused by fungi and yeast affect animals including humans and plants worldwide. There exist a large number of such organisms which can contaminate water and food supplies and cause infections in various body tissues and fluids.

Conventional methods to detect the presence of contaminating fungi, yeast and insects require obtaining samples from an animal or plant suspected of containing these organisms and culturing the samples to grow the desired organisms present in the sample in sufficient numbers to readily detect their presence visually. Typically, culturing the organisms requires specialized media and lengthy culture times of up to several weeks.

Other methods involve the use of a hot basic solution such as a 20% potassium hydroxide to clear smears of specimens on a solid substrate. The cleared specimen is then stained, for example using India ink, then examined by microscope to detect the presence of fungal structures remaining after this treatment.

Fungi, yeasts and insects contain certain substances, including proteins, that may be specific for a particular species. Other substances are more widely distributed. For example, chitin (N-acetylglucosamine oligomer) is a polysaccharide component of cell walls found in most fungi, yeasts and insects and is reactive with reagents such as lectins (Galun et al., Arch. Microbiol., 108(1):9-16 (1976)).

Assays for the presence of chitin-containing organisms such as fungi are known but have been limited to the detection of chitin using chemical analyses including colorimetric determination (Sharma et al., Trans. Br. Mycol. Soc., 69(3):479-83 (1977)) and the use of nitrous acid-3-methyl-2-benzothiazolinone hydrazone hydrochloride-ferric chloride and light microscopy (Kaminskyj et al., Can. J. Bot., 60(12):2575-80 (1982)). Immunological assays to detect various microorganisms including fungi using antibodies are also known, including those employing monoclonal antibodies reactive with antigens associated with particular organisms. (Goldstein, European Patent Application EP 176,355 (1986)). Such assays are based on species-specific proteins. In addition, to detect the organism the assays typically require that the specific protein be isolated or exposed for reaction with the antibody in the assay.

Labeled chitinase has been reported to be useful for visualization of chitin with electron and fluorescence microscopy. Chamberland et al., Histochemical Journal, 17:313-321 (1985), reported the use of a chitinase-gold complex to localize chitin ultrastructure in tomato root cells infected by Fusarium sp. to study host-pathogen relationships. Molano et al., J. Cell Biol., 85:199-212 (1980), reported the use of chitinase-gold complex to study the distribution of

chitin in Saccharomyces cerevisiae. Both techniques require the use of an electron microscope for visualization. Molano et al. also reported the use of fluorescein isothiocyanate (FITC)-chitinase to visualize chitin with fluorescence microscopy.

Hadwiger et al., Plant Physiol., 67:170-175 (1981), reported the use of anti-chitosan and anti-fungal cell wall antisera to study the localization of fungal components in pea-Fusarium interaction. The antisera were conjugated with FITC. Therefore, to detect chitin in the tissue, the samples had to be examined with a UV microscope. Embedding and immunochemical staining of sections were also prepared for transmission electron microscopy. Procedures such as described by Chamberland et al., Molano et al., and Hadwiger et al. necessitate microscopy to visualize the chitin in the sample. These procedures usually were accompanied by elaborate sample preparation methods.

U.S. application serial no. 426,538, filed October 24, 1989 (incorporated herein by reference), disclosed a method of detection of fungi, yeasts and insects without requiring in vivo or in vitro culturing techniques or complex staining reagents, which method relied on lectin or chitin antibodies to target the chitin. The present invention offers substantial improvements over this method.

Summary of the Invention

The basic inventive concept of the assay methods and means for carrying out those methods is based on the recognition that certain organisms including fungi, yeasts and insects are rich in chitin. Accordingly, the assay of the invention is for the direct detection of chitin and by implication deducing the

presence of the chitin-containing organisms. An essential feature of the present invention is that it involves the use of enzymes which specifically bind to chitin. Such enzymes are referred to as chitin-binding biological reagents and chitin-binding or chitin-specific enzymes and include chitinase and lysozyme. Such chitin-binding enzymes are directly or indirectly attached to a label before or after they bind to chitin. Thereby the assays of the invention can be used to selectively attach detectable labels to chitin present within a sample.

The assay of the invention makes it possible to detect chitin by the use of enzymes which specifically bind chitin. Although the invention can be described in terms of several different specific embodiments, such specific embodiments can be generally classified under two subgeneric embodiments. More specifically, in accordance with one subgeneric embodiment, an enzyme which specifically binds chitin, such as chitinase, is bound to the surface of a support such as the bottom of a microtiter plate well, the surface of a glass slide or the surface of a dipstick. Samples to be tested are then brought in contact with the enzyme, bound to the surface and various procedures are carried out in order to determine and detect the presence of any chitin binding to the enzyme on the surfaces. In accordance with a second subgeneric embodiment, a fluid sample or a fluidized sample is filtered through a membrane in order to capture the solid components of the sample on the surface of the membrane. Accordingly, any chitin-containing organisms, chitin, or fragments thereof within the sample become bound to the membrane surface. The bound sample is then contacted with an enzyme capable of selectively binding to any chitin in the sample, for example chitinase or lysozyme. The chitin-binding enzyme

may be directly or indirectly labeled to permit detection. The presence of the chitin-containing organisms such as fungi is determined by visually detecting the label with the naked eye.

A primary object of the present invention is to provide a rapid detection assay which uses a chitin-binding enzyme for determining the presence of chitin-containing organisms without the need for in vitro or in vivo culture techniques or time-consuming staining procedures.

A feature of the present invention is that the assay detects the cellular chitin component of all organisms containing chitin such as fungi, yeasts and insects, which chitin component is generally absent in bacteria and mammalian and plant tissue, reducing possible false positive readings.

An advantage of the present invention is that it can be used to detect chitin and deduce the presence of chitin-containing organisms in samples of all types of biological (mammalian and plant) fluids and tissues as well as food and water without the need for any culturing techniques or staining procedures. The method is not limited to particular species of fungi, yeasts or insects since all such organisms contain chitin.

Another advantage of the present invention is that it provides an efficient, economical, clinical laboratory assay technique for the rapid diagnosis of fungal infections in patients.

Another feature of the present invention is that it provides an assay procedure which can be used by agricultural and food laboratories to evaluate field samples for the presence of fungi, insects, and related pathogens and contaminants.

Another object of the invention is to provide a portable (preferably disposable) diagnostic kit which can be used to detect the presence of chitin-containing organisms in an efficient and economic manner.

Another feature of the present invention is that the fungal contaminants do not have to be directly cultured nor do they require the use of classical chemical staining procedures for the localization of the contaminants.

Another feature of the present invention is that neither lectins nor anti-chitin antibodies are used to detect chitin which is a desirable feature in that lectins will often bind nonspecifically to nonfungal carbohydrates other than chitin, whereas anti-chitin antibodies are undesirable in that they are, at best, difficult to generate due to chitin's poor immunogenicity.

An advantage of the present invention is that the detection means do not require the use of either electron or fluorescent microscopy to effect detection.

These and other objects, advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the structure and usage as more fully set forth below, references being made to the accompanying figures forming a part hereof.

Brief Description of the Drawings

The present invention will be described in connection with the accompanying drawings in which:

Figure 1 is a schematic view of an embodiment of the invention wherein the chitin is bound on a permeable membrane matrix;

Figure 2 is a schematic view wherein the chitin-containing sample is present in a coated microtiter well;

Figure 3 is a schematic representation of the components of an assay scheme which includes a membrane support, chitinase, rabbit anti-chitinase antibody, and alkaline phosphatase conjugated to goat anti-rabbit IgG antibody, which enzyme can generate a colored product from a chromogenic solution;

Figure 4 is a schematic representation of the components of another assay embodiment of the invention wherein the porous membrane captures the chitin, a chitin-binding enzyme attaches to the bound chitin, thereafter a conjugate comprised of a signal generating enzyme bound to an antibody reactive with the chitin-binding reagent binds to the chitin-binding reagent and a chromogenic solution is added, which solution contains the substrate for the signal generating enzyme, which substrate releases a color when contacted with the enzyme;

Figure 5 is a schematic representation of the components of yet another embodiment of an assay of the invention which includes a support having bound thereto chitin having bound thereto avidin-conjugated chitin-binding biological reagent having bound thereto biotinylated enzyme and a chromogenic solution;

Figure 6 is a schematic view of the components of yet another embodiment of the assay of the invention wherein a solid support has thereon chitin which has bound thereto an enzyme-conjugated chitin-binding biological reagent wherein the enzyme of the conjugate will react with its substrate in a chromogenic solution to form a colored product; and

Figure 7 is a schematic view of the components of yet another embodiment of the assay of the invention which includes a chitin-binding biological reagent which is chitinase coated on a solid support, chitin bound to the coated support, chitin-binding biological reagent bound to the chitin, rabbit anti-chitin-binding reagent antibody bound thereto, an enzyme conjugated to goat anti-rabbit IgG antibody and a chromogenic solution.

Detailed Description of the Preferred Embodiments

Before the present method of detecting chitin-containing organisms such as fungi, yeasts and insects and assay devices and kits for carrying out such are described, it is to be understood that this invention is not limited to the particular methods, assay devices or kits described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

It must be noted that as used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an enzyme" includes mixtures of enzymes, reference to "a sample for testing" includes reference to mixtures of such samples and reference to "the method" includes one or more of the methods described and so forth.

The method of the present invention allows the rapid detection of the presence of a variety of chitin-containing organisms including fungi, yeast and insects, without requiring costly and time-consuming culturing or

staining of the organisms. The method provides an assay for detecting chitin using chitin-specific enzyme reagents and is not limited to any particular strain or species of organism. In addition, the method does not require that specific substances such as proteins or carbohydrates be isolated from a sample suspected of containing organisms, prior to detection in the assay.

The substance that is detected in the method of this invention is chitin, an N-acetyl-glucosamine oligomer present in the cell walls of nearly all fungi and yeasts as well as the exoskeleton of many insects and other arthropods.

To carry out the invention, a sample suspected of containing target organisms is obtained. The sample may include a fluid and non-fluid component and may originate from humans, animals, plants or food items. Typical samples include biological fluids such as urine, spinal fluid, whole blood or serum. Other samples include biological specimens such as skin scrapings, sputum, tissue homogenates, wound exudates, or hair. In addition, samples may consist of materials from plants including tissue, scrapings, fluids, exudates and homogenates, or may be water or food suspected of contamination.

Depending upon the type of sample obtained, different embodiments of the invention are more readily applicable for assaying for the presence of chitin in the sample. Although the invention includes a wide variety of different possible embodiments and different chemical and biological reagents which can be used in connection with each of these embodiments, the invention is generally encompassed by two subgeneric embodiments. In accordance with the first subgeneric embodiment, the sample includes fluid and nonfluid components or is

fluidized so as to contain both components and is thereafter filtered through a porous membrane in order to capture the nonfluid-containing components on the surface of the membrane. The presence of chitin in these nonfluid-containing components is then assayed by the use of an enzyme which specifically binds to chitin. This subgeneric embodiment is generally shown within Figure 1 and specific embodiments thereof are schematically represented within Figures 3-6.

The second subgeneric embodiment of the invention involves coating the surface of a material such as a microtiter well with an enzyme which specifically binds to chitin. Once the enzyme such as chitinase has been bound to the surface, the sample to be assayed is brought into contact with the bound chitinase for a sufficient period of time to allow binding to occur between any chitin in the sample and the chitinase bound to the surface. This subgeneric embodiment of the invention is shown within Figure 2. A specific embodiment schematically demonstrating how this subgeneric embodiment can be carried out is shown within Figure 7. However, it should be noted that by various manipulations which will be apparent to those skilled in the art, the subgeneric embodiment shown within Figure 2 can also be carried out using the specific embodiments shown within Figures 3-6, i.e., it need not be carried out only using the specific embodiment of Figure 7.

When the chitin-specific enzyme is to be adhered to a substrate surface, it may be adhered to the surface of a glass slide, microtiter well, dipstick or other suitable material. When the sample is to be filtered through a membrane, any suitable membrane filter can be used, such as those composed of various synthetic or natural polymeric materials.

The two subgeneric embodiments of the invention are shown in figures 1 and 2. In figure 1, the solid components of a sample comprised of liquid and solid components have been attached to a membrane matrix. Various specific embodiments are shown in figures 3-6. The surface of the matrix is preferably designed such that it will hold the solid components and particularly the chitin within the sample securely in place on the surface of the matrix. It is often desirable to include a blocking agent (described further below) to protect active binding sites that have not been occupied by chitin. After the sample has been secured to the matrix, different procedures are carried out whereby a signal (preferably a visually-observable color signal) is generated and the presence or absence of chitin is determined and thereby the presence or absence of chitin containing microorganisms are determined.

The sites on the surface of the membrane which do not have chitin bound thereto are preferably blocked, for example by incubation of the fixed sample on the membrane with an irrelevant protein solution such as bovine serum albumin (BSA), casein or egg albumin. Blocking reduces any nonspecific interactions (i.e., electrostatic interactions) of the binding sites which may interfere with the assay to detect chitin.

In accordance with the embodiment shown in figure 2, the surface of the well is coated with a chitin-specific enzyme. A blocking agent is then applied to occupy all remaining binding sites of the well. A sample which may contain chitin is placed in the treated well surface. A specific embodiment is shown in figure 7. Chitinase is added and allowed to incubate with the sample for a time period in the range of about 3 minutes to about 1 hour to allow the chitinase to bind to any

chitin present within the sample. The wells are then washed with a buffer to remove any chitinase which is not bound to chitin bound to chitinase on the surface of the microliter well. After the washing, any immobilized chitin on the well plate would be detected by adding a reagent which causes a color reaction after contacting a label connected directly or indirectly to the chitinase. This methodology can be used in connection with both insoluble and soluble signal detection strategies. That is, if an insoluble signal reagent is used, the signal will appear coated on the bottom and sides of the well. If a soluble signal reagent is used, the supernatant can be read visually with the naked eye in situ or by highly reliable and commonly used automatic plate readers, or drawn off for processing in a separate detector.

The sample can also be manually smeared onto a glass slide with a cotton swab, scalpel blade, or other similar device, allowed to dry completely and then fixed with chemicals. Heat may also be used for fixation, for example, the solid phase may be placed over a boiling (100°C) water bath until the sample adheres onto the solid phase.

If the sample has no fluid component, it may be manually placed on the solid phase and adhered using chemical fixatives, or it may be suspended in a fluid and applied to a solid phase as described above.

The assay methodology of the present invention is based on the use of the chitin-specific enzyme which is directly or indirectly attached to a label. Accordingly, the assay of the invention can be used to detect chitin-containing organisms present in situ on a surface without having to remove or destroy the surface on which the organisms might be present. For example, if one suspects a plant to have a fungal infection on its surface,

a biological reagent of the present invention containing a chitin-specific enzyme can be sprayed onto the surface of the plant. Thereafter, washing should be carried out in order to wash away any unbound chitin-specific enzyme. After washing, various procedures such as those shown within the specific embodiments of Figures 3-7 can be carried out in order to detect any chitin-specific enzyme bound to the chitin which might be present on the plant. Such a method might be carried out by using chitinase bound to another enzyme which generates a color when placed in contact with a chromogenic solution. Such a two-enzyme conjugate could be contacted with the plant surface, followed by washing, followed by the application of a chromogenic solution. If the surface changes color, such would indicate the presence of a chitin-containing organism on the plant surface.

The assay of the invention relies upon binding of a chitin-binding enzyme reagent specifically binding to chitin and the visualization of this binding by a labeling system. The binding reagent is preferably chitinase.

Polyclonal antibodies reactive with chitinase may be obtained by recovery of serum-containing antibodies following immunization of a mammalian host using chitinase as the immunogen. Procedures for producing polyclonal antibodies are well known and will not be repeated here. The serum-containing polyclonal antibodies are used for binding to chitinase which is bound to chitin in an assay described below to detect chitin-containing organisms.

Monoclonal antibodies reactive with chitinase may also be used in an assay to bind to chitinase. These monoclonal antibodies may be derived using known techniques following the procedures of Kohler and Milstein,

Nature, 256:495 (1975), incorporated herein by reference, to disclose methods of obtaining monoclonal antibodies. In this procedure, hybridomas are prepared by fusing antibody-producing cells (typically spleen cells of mice previously immunized with an antigen) to cells from an immortal cell line such as myeloma cells, using somatic cell hybridization.

Although it has been found that chitin is substantially nonimmunogenic and that, therefore, it is difficult to generate chitin antibodies, it has been found that anti-chitinase antibodies can be generated and that such anti-chitinase antibodies are useful in accordance with different embodiments of the present invention. Polyclonal and/or monoclonal antibodies which are anti-chitinase antibodies are useful in carrying out different specific embodiments of the invention. We have generated polyclonal anti-chitinase antibodies in rabbits, which antibodies can be used in the detection of chitinase. Accordingly, if chitin is bound to a support surface and chitinase is added and then attaches to the chitin, these anti-chitinase antibodies will bind to the chitinase. Such anti-chitinase antibodies are generally labeled, such as with an enzyme, which enzyme generates a color when brought into contact with a chromogenic solution.

Polyclonal anti-chitinase antibodies were generated using two New Zealand White rabbits. The rabbits were immunized with 1 mg of chitinase from Streptomyces griseus (obtainable from the Sigma catalog C1525) present in Ribi adjuvant. The rabbits received a second immunization three weeks later and were then immunized twice more at two-week intervals. Serum was collected from the rabbits and assayed for anti-chitinase antibodies by ELISA. The results obtained showed that

both of the rabbits possessed high serum titers of anti-chitinase antibodies. Resulting serum obtained from the animals was stored at -30°C and used in connection with assays such as those described below.

Anti-chitinase antibodies are believed to be novel and therefore constitute another aspect of applicants' invention. Further, conjugates formed between anti-chitinase antibodies and enzymes capable of generating a colorant upon contact with a chromogenic solution include another aspect of applicants' invention. It should be pointed out that the anti-chitinase antibodies can be conjugated to other enzymes either directly or indirectly. As an example of such indirect conjugation, other antibodies with respect to the anti-chitinase antibodies can be generated, which antibodies are connected to a label or enzyme capable of generating a color upon contact with a chromogenic solution.

The assay to detect the presence of chitin-containing fungi, may be a direct binding assay. In such an assay the chitin-binding enzyme reagent, chitinase or lysozyme, is reacted with chitin in fungi present in the sample, by contacting the sample attached to the solid phase with a labeled binding reagent. The label is then visually detected to determine whether fungi are present.

The presence of fungi may be quantified, by using predetermined amounts of the labeled assay reagent and relating the intensity of the signal produced by the label (which is a function of the amount of assay reagent reacted), to the concentration of chitin using standard binding curves. These curves are generated by measuring the intensity of the signal produced using known amounts of chitin.

The amount of chitin may also be quantified using an indirect, competitive inhibition assay in which

a mixture of labeled assay reagent, for example chitinase, and organism-containing solution is mixed and then added to react with chitin attached to the solid support. Chitin-containing organisms will compete with the immobilized chitin and thus reduce the degree of signal produced by the label in a dose-related manner, permitting a determination of the amount of chitin present in the organisms in the sample.

Visualization of the binding of the assay reagent to chitin present in the sample, may be accomplished by directly labeling the chitinase directly or indirectly with a substance capable of producing a signal, for example, a radionuclide, enzyme or a fluorescent agent, using known procedures. If an enzyme label is employed, an enzyme is selected which when reacted with its appropriate chromogenic reagent produces a color or other visibly detectable signal. In those instances where the enzyme substrate is to be used in solution to contact the enzyme-labeled reagent bound to the sample on the solid phase, a soluble enzyme substrate such as orthophenylenediamine (OPD) reactive with the enzyme horseradish peroxidase (HRP) may be used.

In accordance with another method the sample or specimen is applied manually to a solid phase such as a glass slide to be visualized. A substrate such as diaminobenzidine or 3-amino-9-ethyl-carbazole and the HRP is used. The colored, insoluble reaction product from the cleavage of the substrate by the enzyme will be deposited at or near the location of the enzyme. Thus when a fungal structure is coated with enzyme-labeled chitinase, the enzymatic reaction (of the enzyme label) will deposit the colored substrate around and on the fungal structure and allow the fungus to be readily detected by visual examination.

Alternately, the chitin-binding enzyme assay reagent may be indirectly labeled, attaching the signal-producing label to an additional substance which binds to the assay reagent. For example, where chitinase is used to detect the chitin, an anti-chitinase antibody may be conjugated with a label and bound to the chitinase for reacting with the chitin. This assay may provide a more sensitive assay for the detection of the organism because more label can be bound per unit of assay reagent.

Biotin/avidin reagents may be used to accomplish binding. In this case, biotin is covalently bound to antibody, for example, anti-chitinase antibody. The biotin-specific receptor protein avidin is conjugated to a signal-generating enzyme, then reacted with biotin to label the antibody. The labeled antibody is then used in an assay to detect chitinase bound to chitin in the sample being assayed.

SPECIFIC ASSAY EMBODIMENTS

The general or subgeneric embodiments of the assay described herein are shown in Figures 1 and 2. Although both of these subgeneric embodiments involve the use of a chitin-binding enzyme such as chitinase, the protocol as shown in Figure 1 involves binding the sample to the substrate first whereas the protocol shown in Figure 2 involves binding chitinase to the substrate first. Regardless of which protocol is used, the chitin within the sample is eventually attached to a label or enzyme capable of generating a colorant thereby making it possible to visually determine the presence or absence of chitin within the sample. The subgeneric embodiment shown within Figure 1 is essentially described in further detail by the specific embodiments shown schematically within Figures 3-6, described in detail below. The

subgeneric embodiment shown within Figure 2 is further described by reference to the specific schematic representation shown within Figure 7. It should be noted that although only one specific representation (Figure 7) is given with respect to the subgeneric embodiment of Figure 2, it is possible to rearrange the various components as per Figures 3-6 to drive specific embodiments of the subgeneric embodiment of Figure 2.

ASSAY OF FIGURE 3

A schematic of a particular embodiment of an assay of the invention is shown in Figure 3. This assay will be described in greater detail than the assays of Figure 4-7, which will be described more briefly in that specific information is given below with respect to Figure 3 which can be applied to the assay shown in Figures 4-7. In accordance with the assay of Figure 3, a sample to be tested for the presence of chitin is placed on the membrane surface such as the porous membrane shown. The chitin in the sample is adhered to the membrane in some manner. This can be done by using any of the above-described methods. It is preferably done by passing the sample through a permeable membrane which acts as a filter and traps solids in the sample such as the chitin.

Once the sample is secured to a porous membrane, it is desirable to block other binding sites on the membrane by the addition of an irrelevant protein solution such as BSA. The use of such blocking reagents can make any of the assay embodiments of the invention more specific. Since the use of blocking proteins and blocking reagents in connection with such assays is known to those skilled in the art, the following description

will not repeat the step with respect to the use of blocking reagents further.

A chitin-binding enzyme, such as chitinase or lysozyme, is added to the sample to be assayed under conditions which allow the chitin-binding reagent to bind to chitin present in the sample. The binding environment may be controlled so that the enzymes can bind to the chitin but not quickly degrade the chitin. Although some enzymatic degradation may occur, the system does not depend on degradation of the chitin to generate a signal and detection ability is maintained even though the chitin may be partially degraded. More specifically, the system takes advantage of the enzyme's own ability to specifically bind to the substrate chitin.

Antibodies to the chitin-binding reagent (e.g., rabbit anti-chitinase antibody) can now be bound to the chitin-chitinase complex in order to provide the first antigen-antibody reaction.

A second enzyme is conjugated to an anti-antibody, i.e., an antibody against the first antibody. For example, alkaline phosphatase is conjugated to goat anti-rabbit IgG antibody. This is added to provide a second antigen-antibody reaction. Finally, a reagent (substrate) for the conjugated antibody is added to generate a detection signal. Substrates for alkaline phosphatase include bromo-chloro-indolyl phosphate and nitro blue tetrazolium in a buffer of aminoethyl propanediol. However, other chromogenic signals can be utilized. Alternatively, the anti-chitinase-binding antibody itself can be conjugated to any label such as an enzyme capable of generating a color in combination with a chromogenic solution. Such enzymes include urease and peroxidase.

ASSAY OF FIGURE 4

The schematic view of the assay shown in Figure 4 includes a support in the form of a porous membrane upon which the sample which putatively contains chitin is placed. The chitin-binding enzyme such as chitinase is then added under conditions such that the chitinase can bind to any chitin present. Extraneous and unbound materials can then be washed away. A conjugate is then added which conjugate is comprised of an enzyme bound to rabbit anti-chitinase antibodies. If chitinase is present in that it is bound to chitin, the antibodies will bind to the chitinase. Unbound material is then washed away. Thereafter, a chromogenic solution is added to provide a color which can be visually detected.

ASSAY OF FIGURE 5

In Figure 5, the chitinase is bound to avidin to form a conjugate. After this conjugate is allowed to bind to the chitin due to the specificity of the chitinase, a biotinylated enzyme is added which is biotin-conjugated alkaline phosphatase. The biotinylated enzyme will bind to the advidin and any bound material can be detected at the addition of the chromogenic solution.

ASSAY OF FIGURE 6

A very simple embodiment of an assay of the invention is shown within Figure 6. In accordance with this embodiment, the essential component is the conjugate comprised of the chitinase bound to an enzyme such as alkaline phosphatase. The chitinase will bind to any chitin present on the support. After the binding, washing takes place followed by the addition of a

chromogenic solution which provides a color if the alkaline phosphatase is present.

ASSAY OF FIGURE 7

The assay embodiment schematically shown within Figure 7 is a specific embodiment of the subgeneric method shown in figure 2. In accordance with this embodiment, a chitin-binding enzyme such as chitinase is first bound to the surface of a solid support, such as a polystyrene support. It is not necessary to bind chitinase to all of the available support surface. Active sites which do not have chitinase bound thereto can be protected by the addition of any suitable blocking agent such as BSA. After the chitinase and blocking agents are added, the sample which might contain chitin is placed on the surface. If chitin is present in the sample, it will bind to the immobilized chitinase. The surface is then washed and more chitinase is added, which will bind to any chitin attached to the surface immobilized chitinase. After allowing the chitinase to bind to the chitin, an anti-chitinase antibody is added which binds to chitinase. After the antibody is allowed to bind, washing is carried out followed by the addition of a conjugate. This conjugate is comprised of an enzyme such as alkaline phosphatase bound to an antibody which binds to the chitinase antibody. For example, such an anti-antibody might be an anti-IgG antibody such as a goat anti-rabbit IgG antibody. After binding takes place and washing has been carried out, a chromogenic solution is added which generate a color after contacting the enzyme such as the alkaline phosphatase.

Although Figure 7 provides the only specific embodiment of the subgeneric embodiment of Figure 2, other specific embodiments encompassed by the general

concept of Figure 2 will be apparent to those skilled in the art upon reading this disclosure and reviewing the specific embodiments shown in Figures 3-6. All of the embodiments include the use of a chitin-binding enzyme, such as chitinase, in order to bind chitin. Different layering techniques involving other reagents such as antibodies and conjugates can be used in different forms and manners in order to obtain different desired effects.

Various conjugates useful in connection with the assays of the invention are also considered to be aspects of the present invention. Different conjugates can be formed using methodology known to those skilled in the art. With respect to the formation of specific antibody to enzyme conjugates, reference is made to the Sigma catalog relating to immunochemicals published in 1991 and to the various publications cited therein. Such publications describe the formation of specific conjugates such as enzyme/antibody conjugates, conjugates formed between alkaline phosphatase and other enzymes or antibodies, conjugates formed between peroxidase and other enzymes or antibodies, as well as conjugates formed between urease and other enzymes or antibodies. Specific descriptions are given with respect to the formation of biotin conjugates. The description contained within the Sigma catalog, as well as the various publications cited in that catalog, are incorporated herein by reference.

An important aspect of applicants' invention involves the anti-chitinase antibodies which are produced in accordance with the methods described above. Various conjugates formed using these anti-chitin antibodies are also considered to be an important aspect of the present invention. Such conjugates can include chitinase/other enzyme antibodies which other enzymes are generally enzymes capable of generating a color when brought into

contact with a chromogenic solution. Anti-chitinase antibodies can also be conjugated to other antibodies which in turn are conjugated to other enzymes which are capable of generating a color upon contact with a chromogenic solution.

As will be apparent to those skilled in the art to which the invention is addressed, the present invention may be carried out by using techniques other than those specifically discussed above without departing from the spirit or essential characteristics of the invention. The particular materials and processes described above are therefore to be considered in all respects as illustrative and not restrictive. For example, labeling agents other than enzymes, such as radionuclides or fluorescing agents, may be used to detect the reagent bound to chitin using procedures known in the art. In addition, samples may be attached to a substrate by other procedures, for example filtration of a fluid sample and centrifugation of materials onto a solid phase. The scope of the present invention is as set forth in the appended claims rather than being limited to the examples of the methods and procedures set forth in the foregoing description.

What is claimed is:

1. A method for determining the presence of chitin in a sample, comprising:

a) attaching a sample suspected of containing chitin to a substrate;

b) contacting the sample attached to the substrate with an enzyme capable of selectively binding chitin; and

c) detecting any enzyme bound to chitin present in the sample, whereby the presence of a chitin is determined.

2. The method as claimed in claim 1, wherein the enzyme is bound to a label visually detectable with the unaided eye to form an enzyme-label conjugate.

3. The method as claimed in claim 2, wherein the enzyme is directly bound to the label.

4. The method as claimed in claim 2, wherein the enzyme is indirectly bound to the label by means of an intermediate compound or antibody.

5. The method as claimed in claim 2, wherein the enzyme is indirectly bound to the label via an anti-chitinase antibody.

6. The method as claimed in claim 2, wherein the enzyme is indirectly bound to the label via an anti-chitinase antibody and an anti-IgG antibody.

7. The method as claimed in claim 1, wherein the enzyme is selected from the group consisting of chitinase and lysozyme.

8. The method as claimed in claim 7, wherein the enzyme is chitinase.

9. The method as claimed in claim 2, wherein the label is an enzyme capable of generating a signal when brought into contact with a chromogenic solution.

10. The method of claim 1 wherein the sample is a biological fluid from a human.

11. The method of claim 10 wherein the biological fluid is selected from the group consisting of urine, blood, sputum and spinal fluid.

12. The method of claim 1 wherein the sample is selected from the group consisting of skin, sputum, tissue homogenates, exudates, and hair.

13. The method of claim 1 wherein the sample is water putatively contaminated with a chitin-containing organism.

14. The method of claim 1 wherein the sample is food putatively contaminated with a chitin containing organism.

15. The method of claim 1 wherein the sample is obtained from a plant putatively contaminated with a chitin-containing organism.

16. The method as claimed in claim 1, wherein the sample comprises fluidized plant tissue putatively containing insects.

17. The method of claim 1 wherein the substrate is selected from the group consisting of filters, membranes, beads, particles, microtiter plates, slides and magnetic particles.

18. The method of claim 1 wherein the substrate is comprised of a permeable polymeric material.

19. A method for determining the presence of chitin-containing organisms in a sample, comprising the steps of:

(a) attaching a sample suspected of containing chitin-containing organisms to the surface of a substrate;

(b) contacting the sample attached to the substrate with an enzyme capable of selectively binding chitin which enzyme is selected from the group consisting of chitinase and lysozyme; and

(c) detecting any enzyme bound to chitin present in the sample, whereby the presence of a chitin-containing organism is determined.

20. The method as claimed in claim 19 wherein the chitin-containing organism is an organism selected from the group consisting of fungi, yeast and insects.

21. A method of determining the presence of chitin in a sample, comprising the steps of:

(a) attaching an enzyme capable of selectively binding chitin to the surface of a substrate;

(b) contacting the enzyme with a sample suspected of containing chitin for a sufficient time to allow any chitin in the sample to bind to the enzyme; and

(c) detecting any chitin bound to enzyme bound on the surface of the substrate, whereby the presence of chitin in the sample is determined.

22. The method as claimed in claim 21, further comprising:

contacting any chitin bound on the enzyme with enzyme capable of selectively binding chitin; and

contacting a conjugate comprised of an antibody conjugated to a label wherein the antibody is capable of selectively binding to the enzyme which selectively binds to chitin.

23. A chitin-detection assay kit, comprising:

(a) chitinase;

(b) an anti-chitinase-binding antibody; and

(c) an anti-IgG antibody conjugated with a signal-generating enzyme.

24. The chitin-detection assay kit as claimed in claim 23, further comprising:

(d) a chromogenic solution which generates a color in contact with the signal generating enzyme.

25. A chitin-detection assay kit, comprising:

(a) lysozyme;

(b) an anti-lysozyme-binding antibody; and

(c) an anti-IgG antibody conjugated with a signal-generating enzyme.

26. The chitin-detection assay kit as claimed in claim 25, further comprising:

(d) a chromogenic solution which generates a color in contact with the signal-generating enzyme.

27. A chitin-detection assay kit, comprising:

(a) an enzyme capable of selectively binding to chitin, the enzyme being selected from the group consisting of chitinase and lysozyme;

(b) an antibody which selectively binds to the enzyme capable of selectively binding to chitin; and

(c) an anti-IgG antibody which binds to the antibody (b), the anti-IgG antibody being conjugated to a signal-generating enzyme.

28. A method for determining the presence of chitin on the surface of a plant, comprising:

(a) contacting the surface of the plant suspected of containing chitin with a reagent containing a chitin-binding enzyme selected from the group consisting of chitinase and lysozyme;

(b) washing the surface of the plant in order to wash away any enzyme not bound to chitin on the surface of the plant;

(c) contacting the surface of the plant with an anti-chitase-binding antibody which antibody is bound directly or indirectly to a signal-generating enzyme;

(d) washing the surface of the plant in order to wash away any unbound signal-generating enzyme; and

(e) contacting the surface of the plant with a chromogenic solution containing a substrate which generates a colored product when brought into contact with the signal-generating enzyme.

29. A method for determining the presence of chitin-containing organisms in the sample, comprising:

a) attaching a sample suspected of containing chitin-containing organisms onto a support base;

b) contacting the sample attached to the support base with chitinase;

c) contacting the chitinase with an anti-chitinase-binding antibody;

d) contacting the anti-chitinase-binding antibody with anti-IgG antibody conjugated with a reagent capable of generating a detection signal; and

e) determining the presence or absence of the signal and thereby deducing the presence or absence of chitin in the sample and the presence or absence of chitin-containing organisms in the sample.

30. The method as claimed in claim 29, wherein the sample is a biological fluid from a human.

31. The sample as claimed in claim 30, wherein the biological fluid is selected from the group consisting of urine, blood and spinal fluid.

32. A conjugate comprised of chitinase bound to the signal-generating enzyme.

33. The conjugate as claimed in claim 30, wherein the chitinase is directly bound to the signal-generating enzyme.

34. The conjugate as claimed in claim 30, wherein the chitinase is bound to an anti-chitinase antibody which is bound to the signal-generating enzyme.

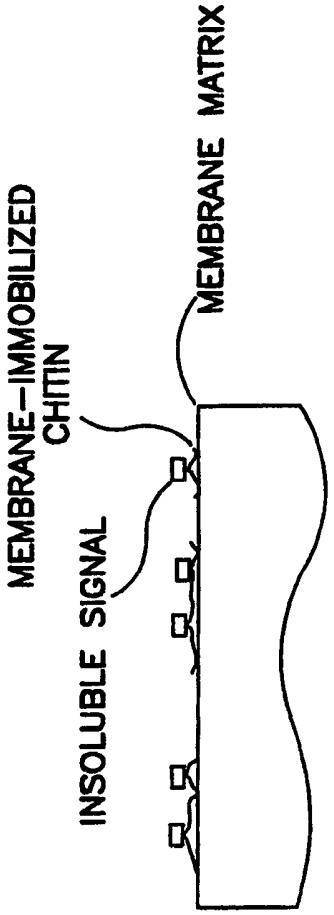

MEMBRANE-IMMOBILIZED CHITIN
INSOLUBLE SIGNAL
MEMBRANE MATRIX

FIG. 1

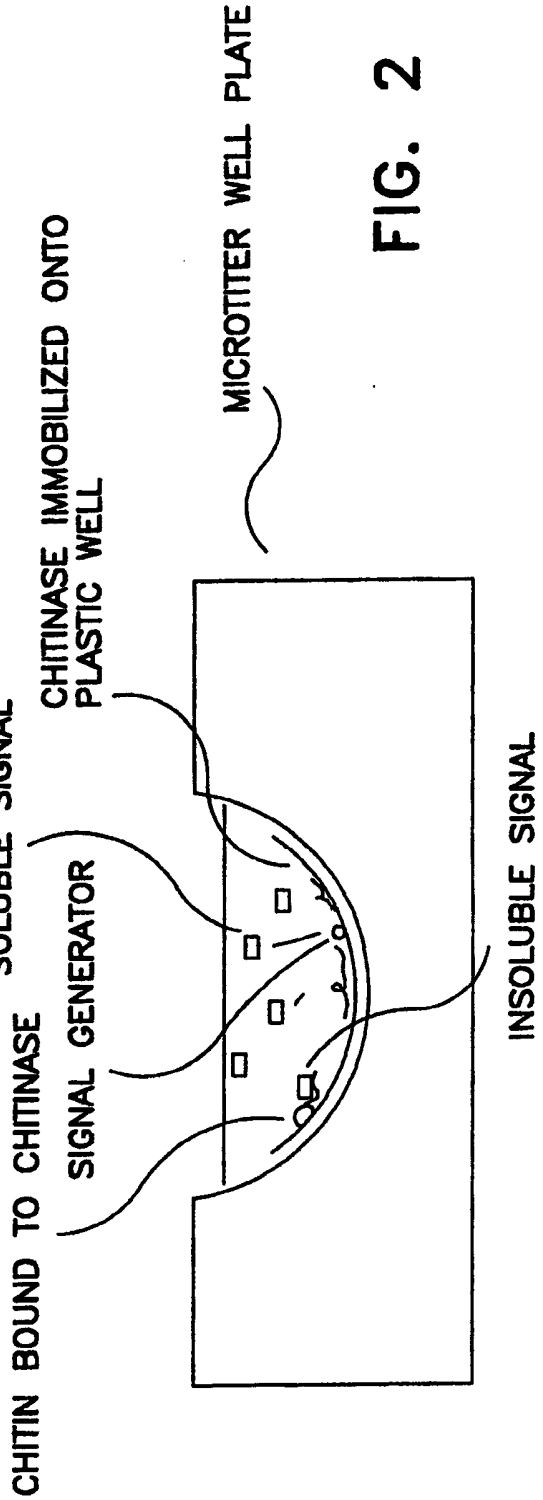
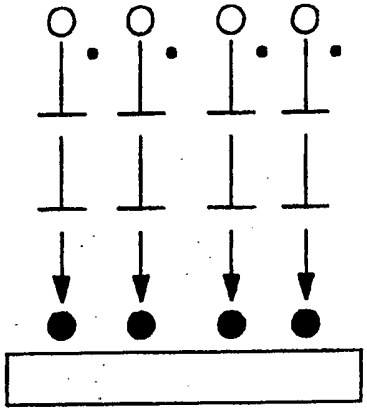

MICROTITER WELL PLATE
CHITINASE IMMOBILIZED ONTO PLASTIC WELL
SOLUBLE SIGNAL
SIGNAL GENERATOR
CHITIN BOUND TO CHITINASE
INSOLUBLE SIGNAL

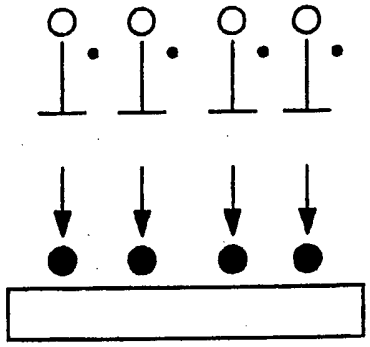
FIG. 2

FIG. 3



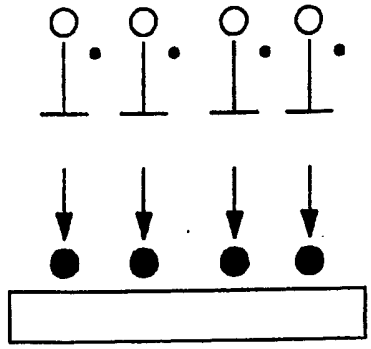
- CHROMOGENIC SOLUTION
- ENZYME CONJUGATED–GOAT ANTI–RABBIT IgG ANTIBODY
- RABBIT ANTI–CHITIN–BINDING REAGENT ANTIBODY
- CHITIN–BINDING BIOLOGICAL REAGENT
- CHITIN
- SOLID SUPPORT–POROUS MEMBRANE

FIG. 4



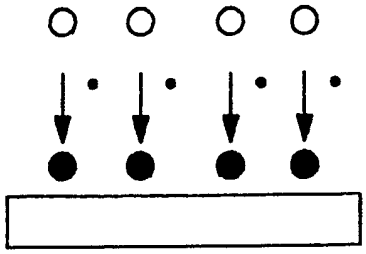
- CHROMOGENIC SOLUTION
- ENZYME CONJUGATED RABBIT ANTI–CHITIN BINDING REAGENT ANTIBODY
- CHITIN–BINDING BIOLOGICAL REAGENT
- CHITIN
- SOLID SUPPORT–POROUS MEMBRANE

FIG. 5



- CHROMOGENIC SOLUTION
- BIOTINYLATED ENZYME
- AVIDIN CONJUGATED CHITIN–BINDING BIOLOGICAL REAGENT
- CHITIN
- SOLID SUPPORT–POROUS MEMBRANE

FIG. 6



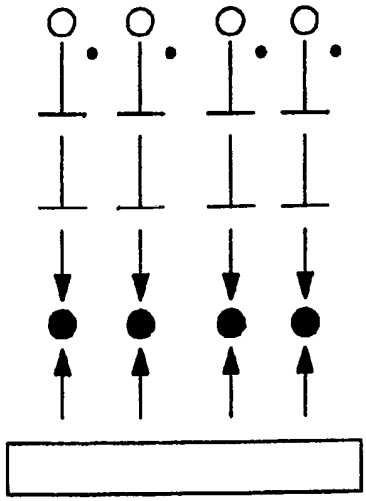
o CHROMOGENIC SOLUTION

ENZYME–CONJUGATED CHITIN–BINDING BIOLOGICAL REAGENT

● CHITIN

SOLID SUPPORT–POROUS MEMBRANE

FIG. 7



o CHROMOGENIC SOLUTION

ENZYME CONJUGATED–GOAT ANTI–RABBIT IgG ANTIBODY

RABBIT ANTI–CHITIN–BINDING REAGENT ANTIBODY

CHITIN–BINDING BIOLOGICAL REAGENT

● CHITIN

CHITIN–BINDING BIOLOGICAL REAGENT

SOLID SUPPORT POLYSTYRENE SURFACE

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/02593

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 G01N33/569; G01N33/573; G01N33/53; G01N33/543

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	THE JOURNAL OF CELL BIOLOGY vol. 85, 1980, pages 199 - 212; J.MOLANO ET AL.: 'Distribution of chitin in the yeast cell wall.' cited in the application see page 199 - page 201 --- -/--	1-3,7,8, 15,17,19

[10] Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 JULY 1992	11. 08. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	HITCHEN C.E.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	THE HISTOCHEMICAL JOURNAL vol. 17, no. 3, March 1985, pages 313 - 321; H.CHAMBERLAND ET AL.: 'Chitinase-gold complex used to localize chitin ultrastructurally in tomato root cells infected by Fusarium oxysporum f. sp. radicislycopersici, compared with a chitin specific gold-conjugated lectin.' cited in the application see the whole document	1-3,7,8, 15,17,19
Y		4-6, 9-14,16, 18,20, 23-34
Y,P	US,A,5 004 699 (M.A.WINTERS) 2 April 1991 cited in the application see column 1 - column 4	4-6, 9-14,16, 18,20, 23-34
Y	Biological Abstracts, Volume 88, Number 3, 1989,Abstract Number 30977, p.Spanu et al., "Chitinase in roots of mycorrhizal Allium porrum: Regulation and localization." See the Abstract. & Planta (Berl.) 177(4): 447-455, 1989.	4-6, 9-14,16, 18,20, 23-34
A	US,A,3 940 317 (F.D.KATZ ET AL.) 24 February 1976 cited in the application see abstract	7
A	EP,A,0 181 851 (S.A.LABORATORIES S.M.B.) 21 May 1986 see abstract	7

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9202593 SA 59266

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 27/07/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5004699	02-04-91	None	
US-A-3940317	24-02-76	DE-A- 2309440	05-09-74
EP-A-0181851	21-05-86	LU-A- 85581	11-06-86